EFFECTS OF INHIBITORY SYNAPSES ON DENDRITIC SPINE CLUSTERING IN ADULT RAT HIPPOCAMPUS

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In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Neuroscience

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Abstract:

Synaptic clustering can serve as a computational unit for the distribution of synaptic resources across dendritic segments. In this study, we investigate whether inhibitory synapses influence the frequency or size of excitatory synapses (whether spine or shaft synapses) in a cluster, and whether the induction of long-term potentiation (LTP) affects this relationship in the stratum radiatum of the CA1 of adult rat hippocampus. We induced LTP through theta-burst stimulation (TBS) in one of two stimulating electrodes through test pulses, while the second stimulating electrode was given test pulses without TBS. We identified symmetric, presumably inhibitory synapses by their equally thin presynaptic and postsynaptic densities, as well as by the pleiomorphic vesicles in the associated axonal bouton. Synaptic clusters were delineated by surrounding asynaptic regions of at least 120 nm. Our preliminary analyses includes 84 clusters and 5 clusters in the LTP and control conditions, respectively. Our preliminary findings show that there are fewer clusters with symmetric synapses two hours post-LTP, and that the surface areas of their symmetric synapses are larger. On one hand, clusters with symmetric synapses had lower asymmetric spine densities than those without. On the other hand, asymmetric synapse densities were consistent between LTP and control if a symmetric synapse is present. These findings suggest that symmetric synapses influences the local spines and synapses in their cluster, and thus serves as an additional layer of analysis in treating synaptic clusters as a computational unit.

Background:

By studying neuronal ultrastructure, we can better understand how the brain functions on a fundamental level. Thus, identifying neuronal subcellular structures as computational units for plasticity is of great interest in neuroscience. Of these structures, we focus on spine clusters of dendrites. Several studies show that learning induces the formation of spine clusters (Fu et al., 2012; Frank et al., 2018; Kleindienst et al., 2011; Bloss et al., 2018). Furthermore, previous work in the mature hippocampus in rats suggests that LTP promotes synapse enlargement while stalling spinogenesis, such that the synaptic weight is consistent across time and condition (Bourne and Harris, 2011; Bell et al., 2014). These findings suggest that spine clustering is a matter of the

redistribution of a finite pool of synaptic resources. Within the dendrite, organelles such as smooth endoplasmic reticulum (SER) and polyribosomes may serve as part of these synaptic resources that promote synapse enlargement. A recent study shows that spine density was lowest in clusters with only resource-poor spines lacking SER and polyribosomes, whereas resource-rich spines preserved their neighboring resource-poor spines in their cluster (Chirillo et al., 2019). Altogether, LTP appears to redistribute synaptic resources in favor of cluster formation.

Interestingly, synaptic weights were consistent across time and condition for both excitatory and inhibitory synapses, suggesting that inhibitory synapses also undergo redistribution after LTP (Bourne and Harris, 2011). This redistribution of inhibitory synapses may occur to modulate the newly formed excitatory connections (Villa et al., 2016). Preliminary cluster analysis from Chirillo et al. (2019) also suggests that spine density is consistent across LTP and control if a symmetric synapse is present. Thus, the presence of an inhibitory synapse may serve as a homeostatic balance to preserve spines in that cluster. Given the role that spine clustering plays in plasticity, learning, and memory, performing a cluster analysis of inhibitory synapses can further our understanding of the criteria needed for cluster formation and maintenance.

Results:

Induction of LTP in the stratum radiatum of the CA1

In addition to investigating how symmetric synapses affect size and number of synapses in a cluster, we aim to investigate if LTP affects this relationship. Theta-burst stimulation was delivered to a stimulating electrode in the stratum radiatum of the CA1 of the rat hippocampus (Fig. 1; Bell et. al, 2014). A second stimulating electrode was given baseline stimulation as a control. For this study, we focus on 2 hours post-LTP.

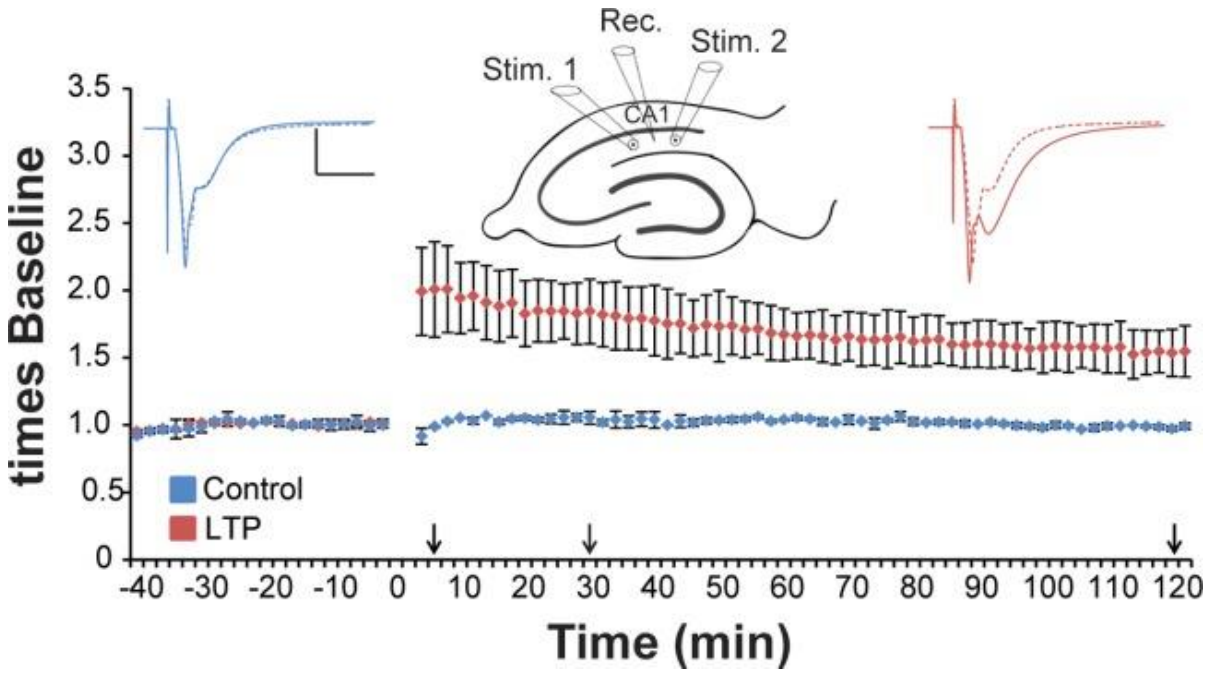

Figure 1: LTP induction in the stratum radiatum of the CA1 of rat hippocampus. A recording electrode was placed in the middle of the stratum radiatum of the CA1, in between two stimulating electrodes. TBS was delivered to one of the stimulating electrodes at time 0 to induce LTP (red) while the second electrode was given baseline stimulation (blue, control). Waveforms were plotted as average responses before (dashed lines) and 2 hours after (solid lines) TBS at one stimulating electrode. [Figure 1 was taken from Bell et. al, 2014].

Identifying symmetric synapses

Serial section electron microscopy was used to identify spines and synapses in our dendrites of interest in the Reconstruct software. To evaluate how inhibitory synapses influence a neighboring cluster of excitatory synapses, we first had to properly distinguish between inhibitory and excitatory synapses. Previous work suggests that excitatory synapses exhibit spherical presynaptic vesicles and an asymmetric pre- and postsynaptic density, whereas inhibitory synapses exhibit flattened or pleiomorphic vesicles with a thin, symmetric pre- and postsynaptic density (Colonnier 1968; Gray 1959). Furthermore, because we expect any individual axon to be exclusively inhibitory or excitatory, we can follow the axon that forms a presumably inhibitory synapse on our dendrite of interest to see if a neighboring bouton also forms an inhibitory synapse (Fig. 2B, 2C). Thus, we used three criteria to identify inhibitory synapses: the presence of pleiomorphic vesicles, the presence of thin (symmetric) postsynaptic densities (PSD), and whether the synapsing axon makes other inhibitory synapses.

Of the three criteria, following the axon to a neighboring bouton is typically the most convincing because the types of synapses for a given axon are usually consistent. For example, an axon whose bouton contains a synapse with pleiomorphic vesicles and a thin PSD is expected to show a similar synapse in another bouton, highly suggesting inhibitory synapses. Furthermore, inhibitory axons tend to synapse onto shafts, whereas excitatory axons tend to synapse onto both spines and shafts. An ambiguous shaft synapse on a dendrite of interest, for example, can be identified as excitatory if its corresponding axon synapses with a neighboring spine. Therefore, following the axon is a powerful tool to verify the identity of a synapse. At times, experimental constraints prevented axons from being followed through serial sections. For instance, the neighboring bouton of an axon may not be visible in the scope of the series. In cases where an axon could not be followed, we evaluated whether the PSD and vesicles of a shaft synapse on a dendrite of interest were conclusive enough to be deemed inhibitory. For example, the same axonal bouton may synapse onto a neighboring shaft with a similar, thin PSD (Fig. 2A). To minimize the false positives of identifying inhibitory synapses, ambiguous shaft synapses were deemed excitatory. In our preliminary work, a total of 12 symmetric, presumably inhibitory synapses were identified across the six dendrites analyzed (Table 1; Fig. 5).

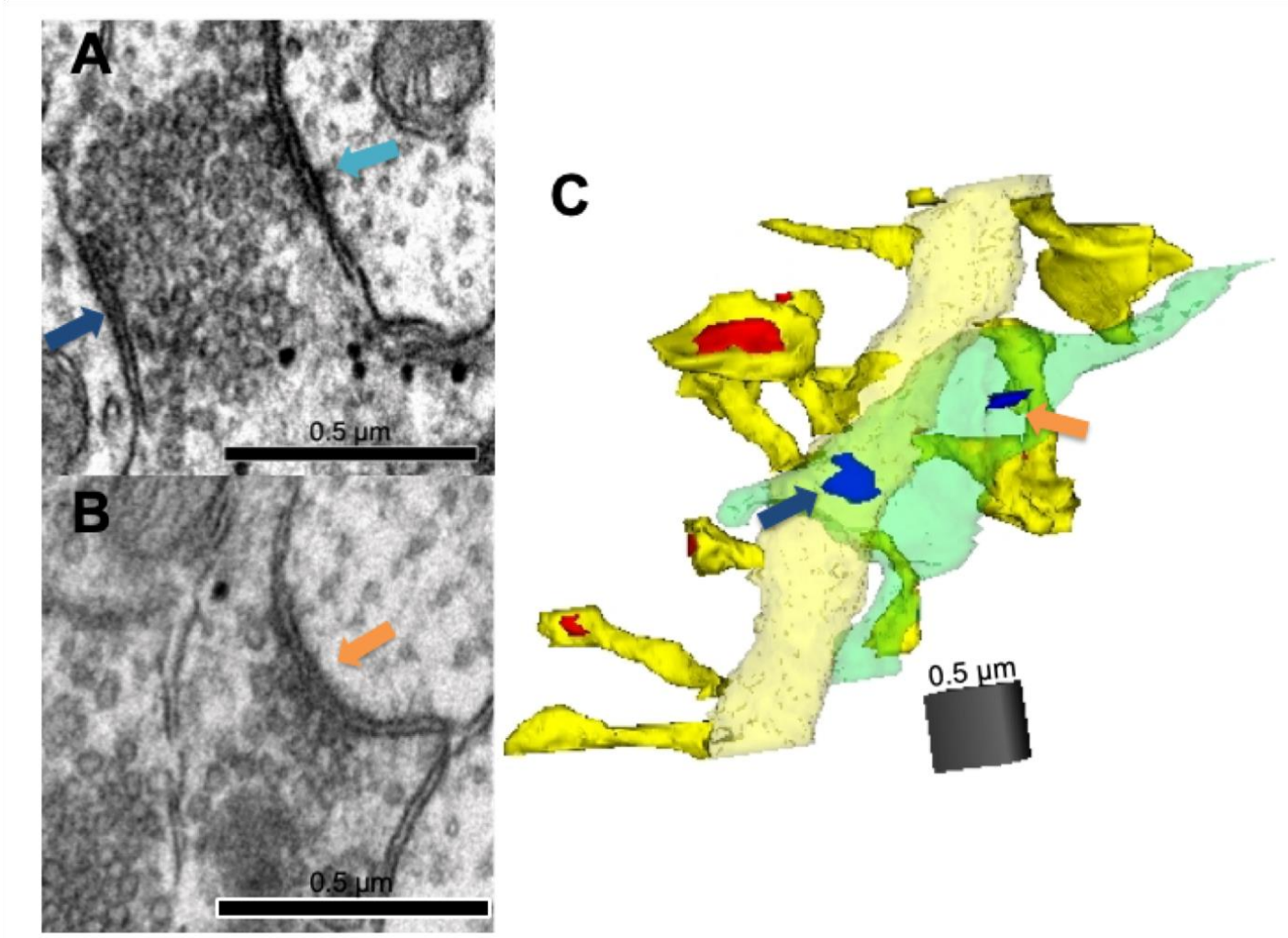

Figure 2: Identification of symmetric synapses. A) Example of a sufficient EM image with a convincing symmetric shaft synapse. Dark blue arrow = inhibitory symmetric shaft synapse on dendrite of interest, light blue arrow = inhibitory symmetric shaft synapse on a neighboring dendrite. Scale bar = 0.5 µm. B) Example of following an axon to confirm a symmetric synapse. The same axon in *A* was followed to a neighboring dendrite to find a symmetric shaft synapse (orange arrow). Scale bar = 0.5 µm. C) Reconstruction of the dendrite of interest and the followed axon. Yellow = dendrite and spines. Red = excitatory synapses. Blue = inhibitory shaft synapses. Dark blue arrow = inhibitory synapse from *A*. Orange arrow = inhibitory synapse from *B*.

Identifying spine clusters

Spine clusters can serve as a computational unit for the sharing of synaptic sources, for previous work shows that spines in a given cluster tend to influence the size and frequency of its neighboring spines in that cluster (Chirillo et al., 2019). To evaluate the role that symmetric synapses play in this spine clustering, we partitioned our dendritic segments into synaptic clusters consisting of spine origins and shaft synapses, and asynaptic regions of at least 120 nm neighboring the cluster (Fig. 3).

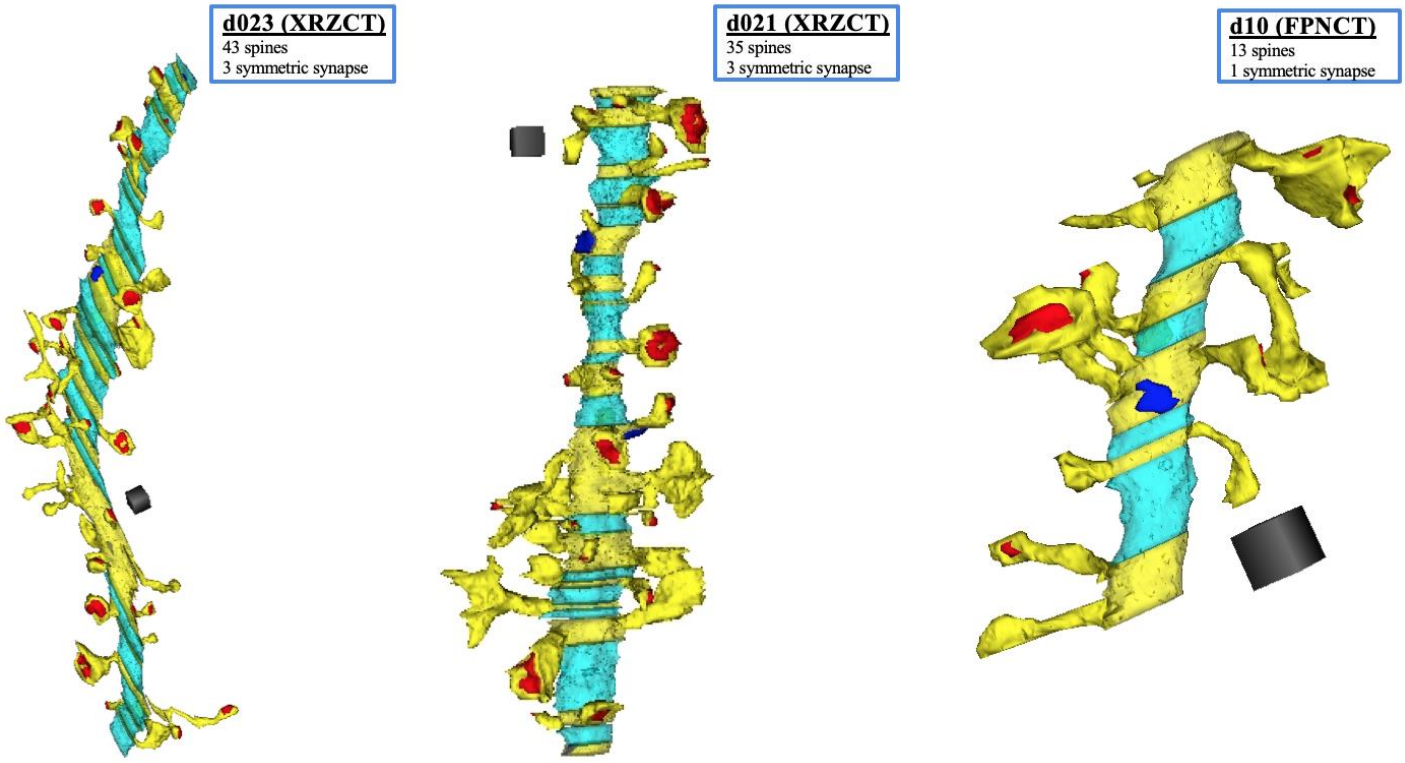

Figure 3: Cluster analysis of dendrites. Yellow = dendrites and spines. Red = excitatory (asymmetric) synapses. Dark blue = inhibitory (symmetric) synapses. Clusters are delineated by asynaptic regions of at least 120 nm (light blue). Scale cube for all dendrites = 0.5 µm each side.

Cluster analysis

To avoid biasing of our traces in the Reconstruct software, we were blinded of the condition (LTP or control) of our dendrites. Another team member who did not trace the dendrites arranged the data gathered to reveal the condition while hiding the identity of the dendrites used. The six dendrites in our preliminary work had 11 clusters with symmetric synapses in the LTP condition. On the other hand, we had only 1 cluster with a symmetric synapse in the control condition (Table 1).

Of the clusters identified, we found that 20% of the clusters in the control group had a symmetric, presumably inhibitory synapse. Conversely, 13.1% of the clusters in the LTP group contained a symmetric synapse (Fig. 4A). Furthermore, the symmetric synapse areas are larger for LTP than that of control (Fig. 4B). Together, these findings suggest

that symmetric synapses are larger but less frequent after induction of LTP. The findings agree with previous work in the lab that suggests a counterbalance between frequency and synapse area after LTP induction (Bourne and Harris, 2011).

We measured the number of asymmetric synapses found in a cluster and the postsynaptic density areas of those synapses, both normalized by length, to evaluate how symmetric synapses influence their size and number (Fig. 4C, 4D). Notably, our preliminary analysis contained four asymmetric, presumably excitatory shaft synapses. To include these shaft synapses in our cluster analysis, we counted all asymmetric synapses in a cluster as opposed to only counting the spines. Our results suggest that asymmetric synapse densities are roughly the same if a symmetric synapse is present in a cluster (Fig. 4C). One possible interpretation is that spines may be preserved in clusters that contain a symmetric synapse. Interestingly, for clusters without a symmetric synapse, the asymmetric synapse density was greater for LTP than control. This finding contradicts our expectations, as we expected LTP to decrease the frequency of synapses (Bourne and Harris, 2011). This discrepancy may be a result of the variance of spine clustering in the stratum radiatum, which can possibly be resolved by compiling our working data with the existing dataset. Furthermore, our findings suggest that, in clusters containing a symmetric synapse, LTP reduces the summed excitatory synapse area of that cluster (Fig. 4D). In clusters without a symmetric synapse, LTP has a greater summed excitatory synapse area.

Importantly, the sample size of the clusters in our LTP condition was greater than that of our control condition (Table 1). Very few clusters (and only one symmetric synapse) were found in the control condition. There may be a wide variance in size and number of synapses in clusters. Our future directions include adding our data to an existing, preliminary dataset in our lab.

	LTP	Control
Clusters	84	5
Symmetric synapses	11	1

Table 1: Identified clusters and symmetric synapses. Counts consist of our preliminary work.

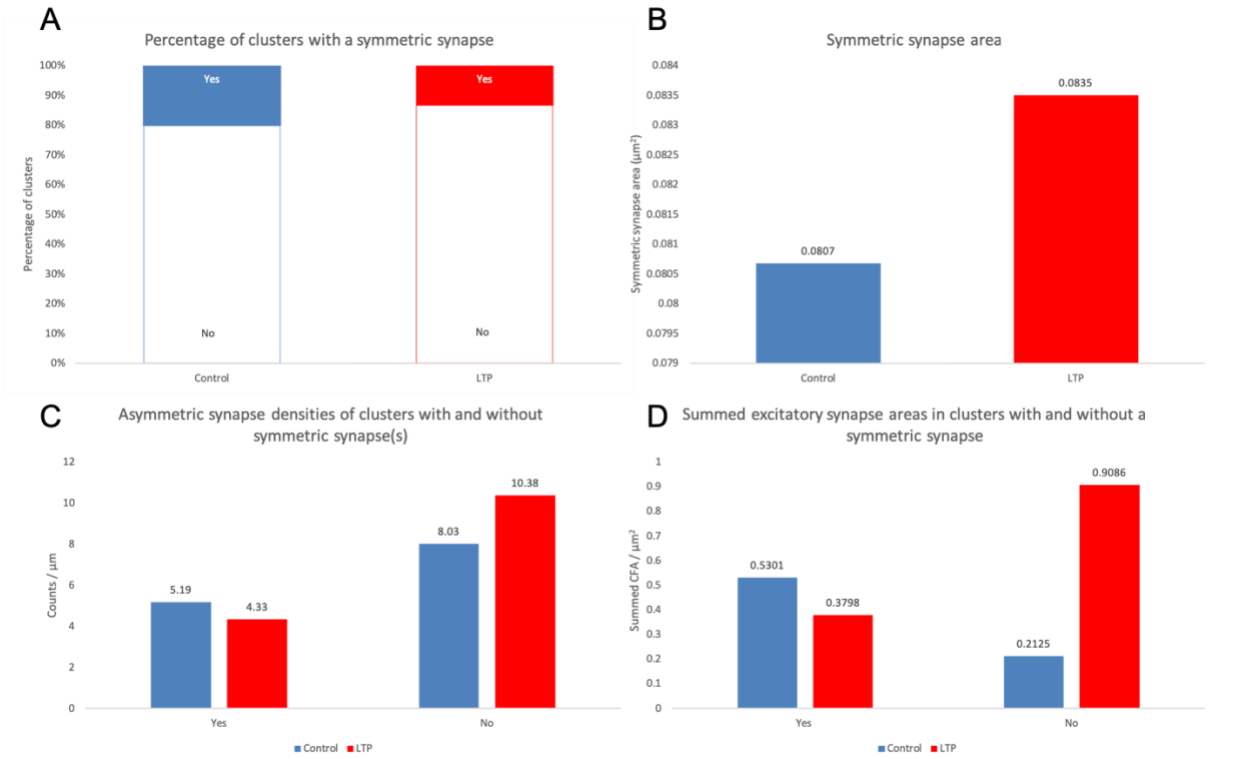

Figure 4: Cluster analysis of synapse areas and densities. A) Percentage of clusters with a symmetric synapse. Blue = control, red = LTP. Color attributes are constant throughout the whole figure. Control = 20% clusters with symmetric synapses, LTP = 13.1% clusters with symmetric synapses. B) Average symmetric synapse area in a cluster (μm^2). Control = 0.0807, LTP = 0.0835. C) Asymmetric synapse densities. For *yes*, SD = 2.14 and SEM = 0.618. For *no*, SD = 4.72 and SEM = 0.538. D) Summed excitatory synapse areas. For *C* and *D*, asymmetric shaft synapses (2 yes, 2 no; LTP) were included in analysis. Six clusters were excluded from analysis in LTP because their spines were incomplete in serial sections.

Discussion:

An underlying principle of our study is using spine clusters as a computational unit for the sharing of synaptic resources. If there is a finite pool of synaptic resources, then synaptogenesis and spinogenesis requires redistribution of these finite resources such that the synaptic weights remain constant (Bourne and Harris, 2011). Thus, clusters may form due to the local distribution of synaptic resources. For instance, recent work on cluster analyses suggests that SER and polyribosomes promote synapse

enlargement and spine clustering after LTP in the adult rat hippocampus (Chirillo et al., 2019). Investigating the role symmetric synapses play in this spine clustering can serve as another layer of analysis on what is necessary for this redistribution of resources. Given this rationale, one future direction for the study is to analyze polyribosomes and SER in our dendrites to see how symmetric synapse clusters interact with the availability of these organellular resources.

Our data suggest that while there are fewer clusters with symmetric synapses after LTP, the density of asymmetric synapses in clusters with a symmetric synapse is consistent across LTP and control. Inhibitory symmetric synapses may preserve spines in their cluster. Additionally, our preliminary findings suggest that the area of symmetric synapses are greater after LTP. These findings agree with previous work in which the both symmetric and asymmetric synapses are enlarged after LTP at the cost of a reduced frequency, suggesting a homeostatic balance of excitatory and inhibitory inputs (Bourne and Harris, 2011). Interestingly, we found that both the asymmetric synapse densities and summed asymmetric synapse areas were greater in LTP than control for clusters without symmetric synapses. This appears to contradict the apparent exchange of frequency for synapse size. However, it is possible that there is great variance in clustering behavior of dendrites, even for within the same condition. It can be qualitatively seen in our clustered reconstructions that there are sections of large and small clusters alike. Qualitatively, there appears to be just as much variation in the length of the asynaptic regions as well. Furthermore, our blinded sampling heavily favored clusters in the LTP condition. In fact, only one symmetric synapse was identified for the control condition. A future direction for this study warrants compiling our analyzed data with that of an existing preliminary data set, which is expected to alleviate the likely variance.

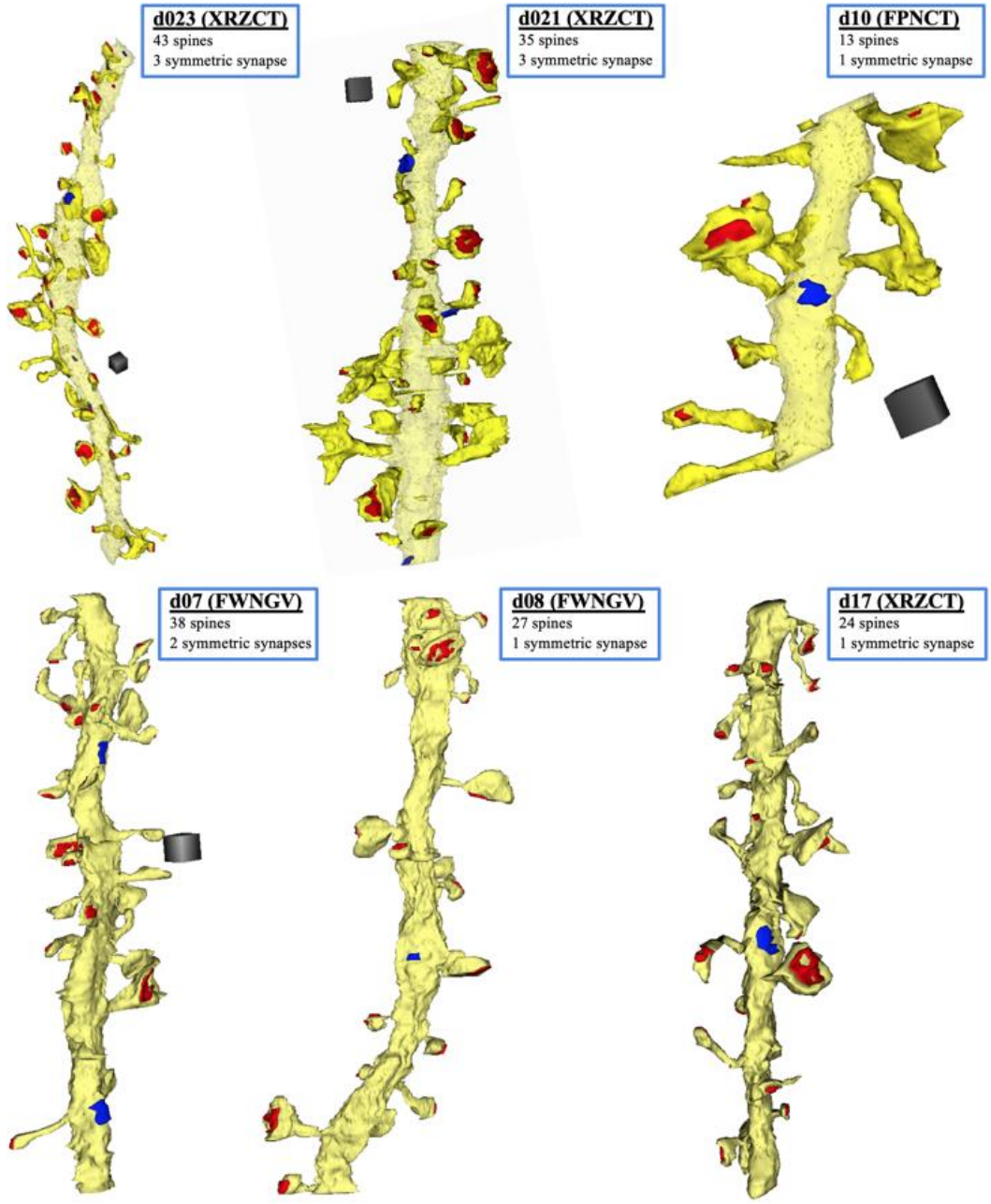

Figure 5: Team-wide reconstructed dendrites. Current dendrites undergoing analysis. Each text box states the dendrite name, as well as the series name in parenthesis. Spine numbers and number of symmetric (inhibitory synapses) are included for each dendrite.

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